

# Isolation of a Factor from Apple that Agglutinates *Erwinia amylovora*<sup>1,2</sup>

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## ABSTRACT

Extracts prepared from apple seeds contain a factor (AF) capable of agglutinating cells of *Erwinia amylovora*. In drop agglutination tests, AF is more active in agglutinating an avirulent, acapsular strain of *E. amylovora* than a virulent, capsular strain. AF precipitates in agar plates with a receptor derived from boiled cells of avirulent acapsular strain and, therefore, can be located during fractionation by rocket electrophoresis. AF was heat-stable and had a pH optimum for agglutination near  $\approx 3.6$  pH. The agglutination activity was not affected by the presence of  $Mg^{2+}$ ,  $Ca^{2+}$ , or EDTA. AF was separated into two fractions (AF I and AF II) by elution from a Bio-Gel P-100 column. The precipitin and agglutination activities associated with AF II were found to be present in a positively charged molecule which was sensitive to treatment with protease and trypsin and, hence, presumably resides in a protein. The approximate molecular weight of AF II was determined to be 12,600 daltons. Besides precipitating the receptor derived from cells of avirulent acapsular strain, AF II was capable of precipitating extracellular polysaccharide from cultures of virulent capsular strain, sodium polygalacturonate, and carboxymethylcellulose. These three polymers also inhibited the agglutination activity associated with AF II. AF II could be replaced by poly-L-lysines in both the precipitin and agglutination assays. In addition, in antigen absorption experiments, poly-L-lysines were found to remove the receptors for AF II from the boiled extracts of avirulent acapsular strain. Based on these observations, it is proposed that the activity of AF II resides in a highly positively charged protein which causes agglutination of bacterial cells by interacting on a charge-charge basis with negatively charged components on the surface of the bacterial cells.

tural data which indicate that the incompatible bacterium *Pseudomonas pisi* and the saprophyte *Pseudomonas fluorescens*, but not the compatible bacterium *Pseudomonas tabaci*, are immobilized in tobacco tissue. Sing and Schroth (19) have found that the saprophyte *Pseudomonas putida* but not the compatible bacterium *Pseudomonas phaseolicola* is immobilized in bean leaves. Finally, Sequeira *et al.* (16) have observed that cells of the avirulent strains of *Pseudomonas solanacearum* become attached to the walls of tobacco mesophyll cells. In contrast, virulent cells remain free in the intercellular spaces and multiply rapidly. These attachment and agglutination phenomena are of interest, first, because they occur early in the interaction and, second, because they, with one exception (4), are observed to affect only the avirulent strain of the microorganism.

There is only limited information available concerning which plant derived molecules are responsible for the selective agglutination or attachment of avirulent strains of bacteria. Sequeira and Graham (7) have shown that the lectin from potato selectively aggregates avirulent strains of *P. solanacearum*. They have also found that a lectin of similar specificity can be extracted from tobacco leaf cell walls. These workers propose that the attachment of avirulent cells of *P. solanacearum* to the surface of tobacco cells results from the specific binding of bacterial cell surface molecules by such a lectin present in the plant cell wall. This binding phenomenon, from which the virulent cells are protected by virtue of their capsule, would be a step unique to the interaction between the plant and the avirulent strain of the bacterium. It could be a decisive step in determining the plant reaction to the microorganism.

Since avirulent cells of *E. amylovora* are agglutinated in the xylem vessels of apple shoots, we began a search for such a lectin or agglutinating factor in apple tissue. In this paper, we describe isolation and partial characterization of this agglutinating factor.

## MATERIALS AND METHODS

**Bacterial Strains.** The virulent, capsulated strain of *E. amylovora*, E<sub>9</sub><sup>4</sup> and the avirulent, acapsular strain, E<sub>8</sub>, have been described previously (1). Lyophilized cultures were stored at  $-20^{\circ}\text{C}$ . Working cultures were initiated from single colonies of correct colony morphology and were checked periodically for bacteriophage sensitivity.

**Media.** Nutrient broth supplemented with yeast extract and glucose, prepared as described previously (18), was the basic liquid (NYGB) and solid (NYGA) medium used. TTC medium, composed of NYGA supplemented with 0.5% (w/v) TTC (11) was

When avirulent strains of phytopathogenic bacteria are introduced into plants, they are physically localized at or near the point of introduction. Such localized bacterial cells are observed either to be agglutinated in the vascular elements (10) or attached to the surface of plant cells (8, 16, 19). For example, Huang *et al.* (10) have shown that cells of the avirulent strain of *Erwinia amylovora* are immobilized in the xylem vessels of apple shoots. Micrographs provided by these workers indicate that electron-dense granules accumulate in the vessels from 6 to 24 h after introduction of the bacterial cells. The granules apparently become attached to the bacterial cell walls and cause their agglutination. Cells of a virulent strain of *E. amylovora* are neither immobilized in the tissue nor agglutinated *in vivo*. Goodman *et al.* (8) have provided ultrastruc-

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<sup>4</sup> Abbreviations: E<sub>9</sub>, virulent capsular strain; E<sub>8</sub>, avirulent acapsular strain; NYGB, basic liquid medium; NYGA, solid medium; TTC, triphenyl tetrazolium chloride; AF, agglutination factor; SAB, sodium acetate buffer; AFR, agglutination factor receptor; EPS, extracellular polysaccharide.

used for determination of colony morphology.

**Procedure for Extraction of AF from Apple Seeds.** Seeds of *Malus sylvestris* X *ranetka* were frozen immersed in liquid N<sub>2</sub> and ground to a fine powder in a Janke and Kunkel grinder, model A 10. The resulting powder (1 g powder/10 ml solution) was extracted with the appropriate extraction solution (detailed below) in a blender at high speed for 5 min. The resulting suspension was filtered through cheese cloth. The insoluble material was re-extracted, filtered, and the two filtrates were combined. The filtrates were centrifuged at 16,000g for 20 min at 4°C. The pellet was discarded, and the supernatant liquid was passed successively through Millipore filters (5, 1.2, and finally 0.45 µm pore size). When the acetone extraction step was included, the filtrate was mixed with 2 volumes acetone at 4°C and stored overnight at the same temperature. The supernatant liquid was discarded, and the white precipitate was suspended in 0.1 M SAB (pH 3.6). One ml buffer was added/g seeds extracted. The suspension was centrifuged at 27,000g at 4°C for 20 min. The supernatant liquid was retained, and the pellet was resuspended in the same amount of buffer and centrifuged again. The pellet was discarded, and the two supernatant liquid fractions were dialyzed against 400 volumes 0.1 M SAB (pH 3.6), at 5°C with continuous stirring. The dialyzed material, termed "crude AF," was stored at -20°C.

Four different solutions were tested for their usefulness in extracting AF from the apple seed meal. They were: (a) The solution recommended by Loomis (13) for the preparation of enzymes from plant tissue. It consists of 0.1 M sodium ascorbate in 0.1 M K-phosphate (pH 7.0). One g insoluble PVP was added/g seed meal. (b) The extraction solution used by Marinkovich (14) as well as Sequeira and Graham (17) and Woods *et al.* (24) for the extraction of lectins from plant tissues. The solution is made by mixing 3 volumes absolute ethanol with 1 volume 1.25 N HCl. One g insoluble PVP was added to each 10 ml of this extraction solution. (c) The solution used by Stokes *et al.* (21) for separation of mitochondria from apple tissue. This solution contains 2 mM sodium metabisulfite in 0.1 M K-phosphate at pH 7.0. (d) This solution consisted of 0.85% (w/v) NaCl in water.

**Extraction of AF from Apple Shoot Tissue.** Apple trees, variety "Red Delicious," were grown in pots under greenhouse conditions. Twenty-five-g lots of shoots 5 to 7 cm in length were frozen in liquid N<sub>2</sub> and ground with a mortar and pestle. The frozen, ground tissue was then extracted in the same manner described for seeds.

**Assay for Agglutination Activity.** Cells of the avirulent strain E<sub>8</sub> were grown on NYGA slants for 24 h at 26°C. The cells were washed from the slants with water and the suspension was adjusted to a concentration of 10<sup>8</sup> cells/ml. Agglutination assays were carried out as described by Sequeira and Graham (17) except that no numerical rating system was used. Reactions were rated as either plus (agglutination) or minus (no agglutination) after a reaction period of 4 h. A plus reaction was a very tight clump of cells in the center of the droplet. An agglutination titer was recorded numerically as the highest dilution of AF solution capable of causing agglutination.

The drop agglutination test was sensitive and linear over a broad range of concentrations of AF. While some error was introduced in determining the titration end point, this variability was found not to exceed ±1 dilution step in the typical 2-fold dilution series used routinely to detect AF.

**Assay for Precipitin Activity.** The AFR used in the precipitin assays was released from E<sub>8</sub> cells by boiling. Cells of E<sub>8</sub> were grown for 24 h on NYGA slants, washed from the slants with 3 ml phosphate-buffered saline (PBS: 0.1 M sodium phosphate buffer [pH 7.0], 0.15 M NaCl), boiled for 10 min, and centrifuged at high speed for 15 min. The pellet was discarded and the supernatant liquid, containing the AFR, was stored at -20°C. One preparation of AFR was used throughout this study.

Precipitin activity in AF preparations was assayed by quanti-

tative rocket electrophoresis (23). Medium electroendosmosis agarose (Miles Laboratories, Inc.), was used at 0.6% (w/v) in 0.1 M SAB (pH 3.6), to prepare plates 5 × 7.5 cm (1 mm thick). A 75-µl sample of the AFR solution was included in each 5 ml agar. Sample wells (2.5 mm diameter) were punched in the sample strip and 4.5-µl samples of the solution to be tested for precipitin activity were placed in the sample wells. Electrophoresis was carried out with a Bio-Rad model 1415 electrophoresis apparatus and a Buchler 3-1500 constant power supply at a constant voltage of 28 v/cm at 25°C for 1 h. At this time, the rockets were fully formed. Rocket areas (mm<sup>2</sup>) were measured with a Rocket Peak Area Estimator from Bio-Rad Laboratories.

This assay was accurate over a 16-fold concentration range of AF. The relationship between peak area and AF concentration had the form  $y = ax + b$  with  $r^2 = 0.99$ . The SD in the value of the area for a series of 10 replicates on a single plate never exceeded ±5%. When it was necessary to compare results from more than one plate, possible plate variation was precluded by including four standard samples of AF (stored at -20°C) on each plate.

**Fractionation of Crude AF Preparation.** A 5-ml sample of the crude AF (end point titer of 512 in the agglutination assay) was fractionated by elution from a Bio-Gel P-100 column (2.5 × 100 cm). The column was prepared in and eluted with 0.1 M SAB (pH 3.6) and 5-ml fractions were collected.

Fractions were analyzed for protein by the method of Bradford (3). Protein concentrations were calculated from a standard curve prepared with BSA. Since no linear relationship could be obtained between AF concentration and OD in this assay, it could only be used for location of protein during fractionation. Total carbohydrate was estimated by the method of Dische (5), and concentrations were calculated relative to a standard of D-glucose. Uronic acid concentration was measured by the method of Bitter and Muir (2) relative to a standard of D-galacturonic acid. Agglutination and precipitin activity of the fractions were determined as described above.

Fractions corresponding to two peaks of agglutination activity (peak I, fractions 13 through 20, and peak II, fractions 40 through 50; Fig. 3) were combined, lyophilized, and redissolved in the elution buffer. These two components will be referred to as AF I and AF II, respectively.

**Treatment of AF II with Proteolytic Enzymes.** *Aspergillus* protease, type XIII, and trypsin, type I (twice crystallized), both from Sigma Chemical Co., were dissolved in 0.1 M SAB (pH 3.6) (8.2 mg/ml) and 25 mM Tricine (pH 8.2) (2 mg/ml), respectively. Serial 2-fold dilutions of each enzyme solution were mixed (100 µl) with 100 µl AF II in distilled H<sub>2</sub>O. Samples of each reaction were removed after 1 h, 3 h, and 24 h. The precipitin and agglutination activity was measured as described above.

**Other Chemicals.** EPS (capsular) was prepared from cultures of *E. amylovora* (E<sub>9</sub>) by the method of Ayers *et al.* (1). Sodium polygalacturonate and poly-L-lysine (types V, VI, and VII-B) were purchased from Sigma Chemical Co. and carboxymethylcellulose was purchased from Hercules Powder Co.

## RESULTS

**Extraction of AF from Apple Seed Meal.** No AF activity was recovered when solution 1 was used, whereas solution 2 provided the greatest recovery of activity. When the acetone extraction step was included in the isolation procedure the agglutination titer of preparations obtained with solution 2 increased from 8 to 256. Therefore, solution 2 plus acetone extraction was adopted as the routine procedure for the preparation of crude AF.

**Specificity of AF Activity in Agglutination Assay.** The ability of the crude AF to cause agglutination of a number of bacterial species was tested. Crude AF preparation was able to agglutinate only strains of *E. amylovora* and was more active in agglutinating the E<sub>8</sub> than E<sub>9</sub>. In experiments with different preparations of crude

AF, the agglutination titer with  $E_8$  was 512 to 1024 and with  $E_9$  it varied from 16 to 32.

**Specificity of AF Activity in Precipitin Assay.** These experiments were first carried out using the double diffusion technique, where crude AF was placed in the central well and extracts from the boiled bacterial suspensions (Table I) were placed in the outer wells. All bacterial suspensions were adjusted to the same optical density ( $A_{500}$ ) before boiling. A precipitin band formed only between the wells containing the crude AF preparation and the  $E_8$  extract. When the experiment was repeated using counterelectrophoresis, all bacterial strains tested, except *Corynebacterium insidiosum*, were found to possess receptor activity for the crude AF. However, much heavier precipitin bands were found with extracts from both strains of *E. amylovora*.

**Characterization of Agglutination Activity Present in Crude AF Preparations.** The pH optimum for agglutination was between pH 2 and pH 4, and therefore, all agglutination and precipitin assays were carried out in SAB at pH 3.6. Raising the pH of a solution of AF from 3.6 to 7 and then reducing it once again to 3.6 caused no loss of either agglutination or precipitin activity.

The agglutination titer of preparations of crude AF was not altered after boiling for 30 min or autoclaving (121 C) for 5 h. All activity was lost if the preparation was charred. The agglutination activity was unaffected by either lyophilization or dialysis. The activity precipitated when subjected to dialysis against distilled  $H_2O$  for prolonged periods (greater than 24 h) but was readily redissolved in buffer. The agglutination titer of crude AF was not affected by 5 mM  $CaCl_2$ , EDTA, or any of the following compounds at the same concentration: melibiose, sucrose, D-xylose, D-mannose, cellobiose, D-galacturonic acid, L-arabinose, lactose, D-glucose, D-galactose, D-fructose, raffinose, D-glucuronic acid, N-acetyl-D-glucosamine, or N-acetyl-D-galactosamine.

**Fractionation of Crude AF by Column Chromatography.** Bio-Gel P-100 fractions of crude AF were assayed for protein, total carbohydrate, uronic acid, agglutination activity, and precipitin activity (Fig. 1). A peak of protein is present in fractions 12 through 24 which was not retained by the column. Only these fractions contained detectable carbohydrate (not shown). A second broad peak of protein was eluted from the column in fractions which preceded those containing most of the material which reacted in the uronic acid assay (not shown).

Two peaks of agglutination activity were detected in the elution profile and they coincided with the presence of protein. A peak of precipitin activity is present between fractions 32 and 52. More

Table I. Specificity of AF Present in Apple Seed Extract in Agglutination and Precipitin Assays

Suspensions of all bacterial species were adjusted to give 1 A at 550 nm (this is equal to  $8 \times 10^8$  colony-forming units with *E. amylovora*,  $E_8$ ). Precipitin tests were carried out on boiled bacterial extracts by counter electrophoresis. A + means that a precipitant line was detectable, a - means that no line was observed, and n.a. means no agglutination. Agglutination assays were carried out as described under "Materials and Methods."

Bacterial Species	Agglutination Titer	Precipitin Test
<i>Agrobacterium tumefaciens</i>	n.a.	+
<i>Corynebacterium insidiosum</i>	n.a.	-
<i>Erwinia aeroidae</i>	n.a.	+
<i>E. amylovora</i> ( $E_8$ )	512	+
<i>E. amylovora</i> ( $E_9$ )	16	+
<i>Erwinia stewartii</i>	n.a.	+
<i>Erwinia uredovora</i>	n.a.	+
<i>Micrococcus luteus</i>	n.a.	+
<i>Pseudomonas pisi</i>	n.a.	+
<i>Xanthomonas malvacearum</i>	n.a.	+

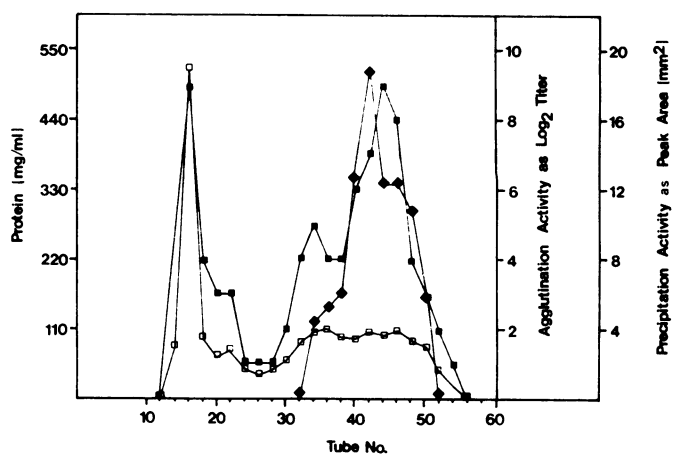


FIG. 1. Profile obtained eluting the crude seed extract from a Bio-Gel P-100 column. Protein ( $\square$ ) is given in mg/ml relative to a standard curve prepared with BSA. Agglutination activity ( $\blacksquare$ ) is displayed as  $\log_2$  of the titer and precipitin activity ( $\blacklozenge$ ) is given in  $mm^2$  area of the rockets obtained after rocket electrophoresis.

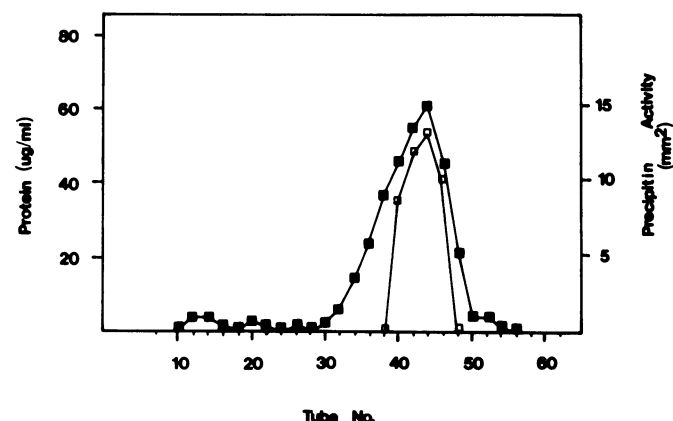


FIG. 2. Profile obtained by re-elution of AF II from the Bio-Gel P-100 column. Fractions 40 through 50 (Fig. 2) were combined, concentrated by lyophilization, redissolved in elution buffer, and re-eluted from the same column (Fig. 1). Protein ( $\blacksquare$ ) is given in  $\mu g/ml$  relative to a standard curve prepared with BSA. Precipitin activity ( $\square$ ) is given in  $mm^2$  area measure from the rockets formed in the rocket electrophoresis assay.

than 95% protein placed on the column was recovered between fractions 12 and 60.

The shoulder of agglutination and precipitin activity present in fractions 30 through 38 was noted in each of the 12 replicate samples. This indicates the possible presence of two agglutination and precipitin activities. No precipitin activity was associated with the agglutination activity present in fractions 12 through 24. While no rockets were formed by compounds present in these fractions, clear precipitin bands were formed at the edge of the sample wells.

**Relationship of AF I to AF II.** To determine whether the two peaks of agglutination activity present in the elution profile (Fig. 1) represent a series of aggregates formed during isolation from a single molecular species, preparations of AF I and AF II were lyophilized, dialyzed, redissolved in elution buffer, and re-eluted from a Bio-Gel P-100 column. The AF I was still not retained by the column (not shown). The elution profile for AF II is given in Figure 2. Coincident peaks of protein and precipitin activity were found in the same fractions as the original elution profile (Fig. 1). If AF I represents a simple aggregate of AF II, then the rate of aggregation was too slow to be detected in this experiment, or other molecules present in the crude preparation are needed to promote or cause aggregation.

**Results Which Indicate That AF II Resides in a Protein.** It was apparent that the AF II activity resided in a molecule with a net positive charge at pH 3.6, because it migrated toward the cathode when subjected to rocket electrophoresis. The actual rate of migration was measured using the Grabar-Williams technique (9). AF II was placed in the sample well and subjected to an electrical field (28 v/cm). At 5-min intervals, electrophoresis plates were removed, a trough was cut, and a solution containing AFR was placed in the trough. Diffusion was allowed to proceed for 1 h in the second dimension and the distance from the sample well to the middle of the precipitant arc was measured. The rate of migration (0.5 mm/min) was linear over the 40-min time course of the experiment.

Both *Aspergillus* protease and trypsin were capable of decreasing the agglutination and precipitation activity present in AF II. The data showing the effect of different concentrations of trypsin on the precipitin activity present in AF II after a 1-h reaction period are given in Figure 3. Boiled trypsin was ineffective. The decrease in precipitin activity was also time-dependent with no precipitin activity remaining after 24 h at any concentrations of trypsin tested. Similar results were obtained in the agglutination assay.

**Determination of Approximate Molecular Weight of AF II.** A Bio-Gel P-10 column (bed volume of 64 ml) was prepared and standardized with dextran blue, myoglobin (horse heart), ribonuclease A (bovine), and insulin (bovine) which have mol wt of  $2 \times 10^6$ , 16,890, 12,640, and 5,733 daltons, respectively. The relationship between molecular weight and elution volume of these standards from the column is described by the equation  $K_{av} = 7.3$  to  $0.74 \ln$  (mol wt) with  $r^2 = 0.99$ . An AF II preparation was eluted from this column and detected with the precipitin assay. The AF II eluted at 33.3 ml which corresponds to an approximate mol wt of 12,600 daltons. Ribonuclease A which has approximately the same molecular weight was eluted from the column in the same fractions as AF II.

**Inhibition of AF II Caused Agglutination and Precipitation by EPS from *E. coli* Cells.** While the capsular strain *E. coli* possessed receptor for AF, the cells of this strain gave a much lower agglutination

titer than the *E. coli* cells. There are two possible explanations for this lower titer: first, the large layer of EPS (1) surrounding the cells in *E. coli*, but absent in the *E. coli*, might physically prevent interaction between the AF and the receptor. Second, the AF may be bound by the EPS, and the large amount of soluble EPS present in the suspensions of *E. coli* might act as a competitor for the receptor on the bacterial cell surface. The second possibility was tested by measuring the ability of EPS prepared from *E. coli* to inhibit the ability of AF to agglutinate *E. coli* and to prevent precipitation of the *E. coli*-derived receptor.

The data presented in Figures 4 and 5 reveal that EPS is able to prevent both the AF II-caused agglutination and precipitation reactions. In the case of the agglutination reaction (Fig. 4), concentrations of 4  $\mu$ g/ml and greater cause a marked decrease in the agglutination titer, and at 128  $\mu$ g/ml EPS agglutination was completely suppressed. Similar inhibition of precipitation activity was observed at EPS concentrations of 128  $\mu$ g/ml (Fig. 5).

The reason for the inhibition of the precipitin assay is clear. When the EPS solution is mixed with the AF II preparation, immediate precipitation was observed. This precipitate is electrophoretically mobile, has a net negative charge, and forms rockets out of the sample wells on the side away from the AFR-containing agar. As expected, precipitate rockets form if AF II is moved, electrophoretically, into EPS-containing agar (Fig. 6). Both carboxymethylcellulose and sodium polygalacturonate are able to inhibit AF II-caused precipitation of AFR. When AF II is moved by electrophoresis into agar containing either of these polymers, rockets similar to those described for EPS are formed.

**Replacement of AF II with Poly-L-lysine.** When all three types of poly-L-lysine were dissolved in 0.1 M SAB pH 3.6 at a concentration of 1 mg/ml, they caused agglutination of *E. coli* cells. These preparations had end point titers near 1000. The preparations of poly-L-lysine also formed rockets when moved by electrophoresis into agar containing AFR.

Poly-L-lysine was also able to remove the receptor for AF II from the AFR preparation. When a 1 mg/ml solution of poly-L-lysine was mixed with an equal volume of AFR solution a precipitate, which could be removed by centrifugation in a clinical

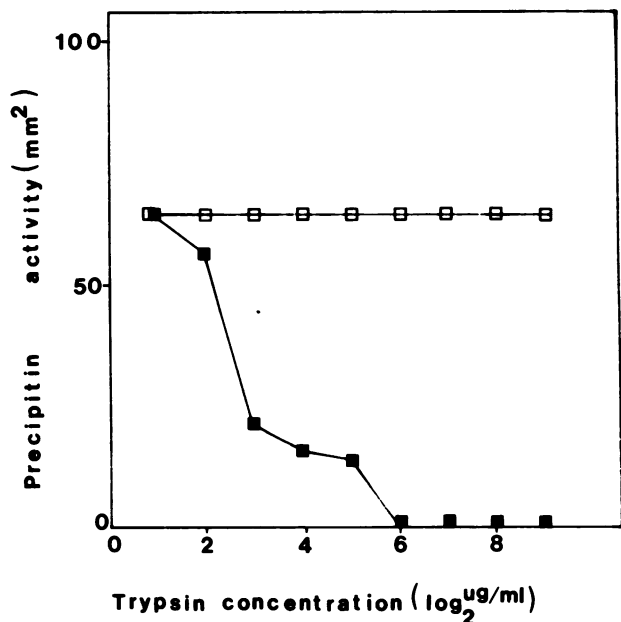


FIG. 3. The effect of trypsin on the precipitin activity in AF II preparations. An AF II preparation capable of forming rockets of 65  $\text{mm}^2$  area was treated with different concentrations of boiled ( $\square$ ) or nonboiled ( $\blacksquare$ ) trypsin. Precipitin activity is given in  $\text{mm}^2$  rocket area and trypsin concentration is given in  $\log_{10}$  of the concentration in  $\mu\text{g/ml}$ . These data were obtained after 1-h reaction period.

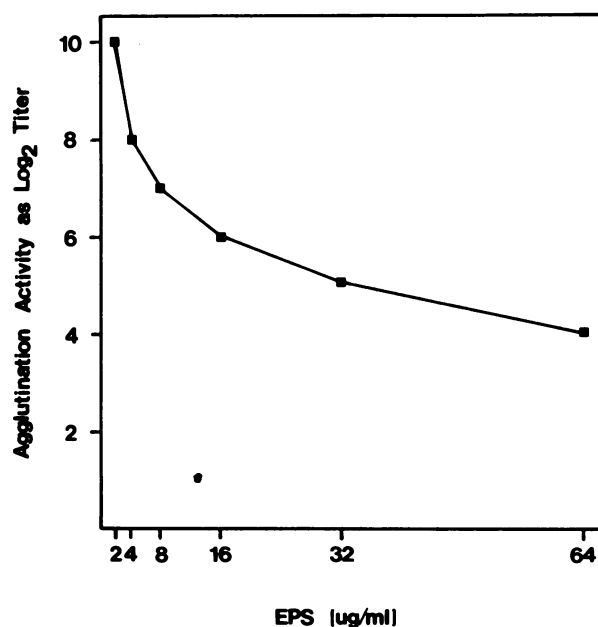


FIG. 4. The effect of EPS derived from cultures of strain *E. coli* on the agglutination activity in AF II. Agglutination activity is given as  $\log_2$  of the agglutination titer. The EPS concentration is displayed in  $\mu\text{g/ml}$ . No agglutination was observed when EPS concentrations of 128  $\mu\text{g/ml}$  or higher were used.

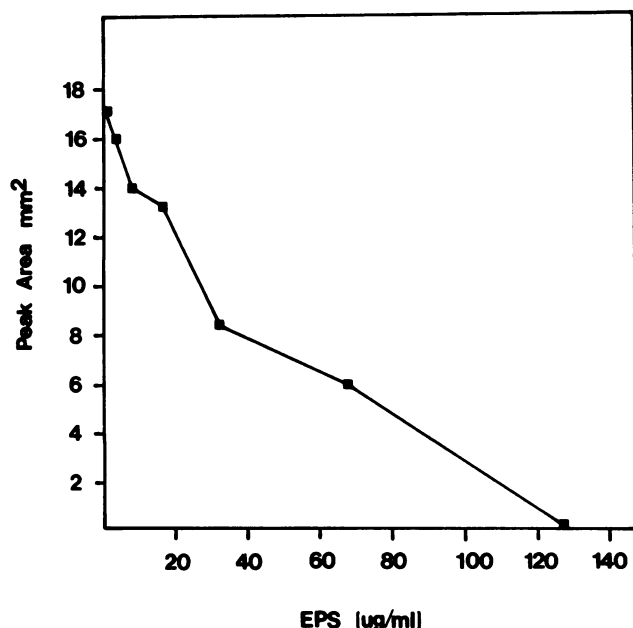


FIG. 5. The effect of EPS derived from cultures of strain *E<sub>8</sub>* on the precipitin activity of AF II. EPS concentration is given in  $\mu\text{g/ml}$ . The precipitin activity measured by rocket electrophoresis is given in peak area in  $\text{mm}^2$ .

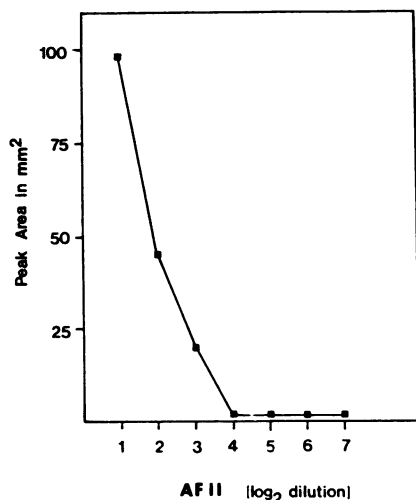


FIG. 6. These data describe the area of rockets formed when AF II was moved electrophoretically into agar containing EPS at a concentration of  $100 \mu\text{g/ml}$ . These data were obtained after 1 h electrophoresis. The rockets were electrophoretically mobile.

centrifuge, was formed. The supernatant liquid no longer contained receptor for AF II as assayed by line electrophoresis into AF II-containing agar (12).

**Isolation of AF Activity from Shoots of Variety "Red Delicious."** Extracts from apple shoots were found to contain an activity which caused selective agglutination of the avirulent strain of *E. amylovora*. As with the seed preparation, use of extraction solution 2 gave the highest recovery of AF. When determined on a fresh weight basis, the agglutination titer of AF preparations from apple shoots never exceeded one-twenty-fifth of the titer from seeds. When the crude AF preparation from shoots was eluted from the Bio-Gel P-100 column, two peaks of agglutination activity were obtained. Here, too, only the second peak of activity was capable of causing both agglutination and precipitin reactions. The elution

profile for the shoot extract was identical with that shown for the seed extract in Figure 1.

## DISCUSSION

The avirulent strain of *E. amylovora* (*E<sub>8</sub>*), when introduced into a cut apple petiole, was localized at or near the point of introduction and appeared to be agglutinated by particles that originated in xylem parenchyma cells (10). The virulent strain (*E<sub>9</sub>*) was neither localized nor agglutinated as it migrated systemically 12 to 15 cm in 24 h.

The purpose of this research was to recover and characterize the factor responsible for agglutinating *E<sub>8</sub>* in xylem vessels. Sequeira and Graham (17) reported that a lectin was responsible for the localization of *P. solanacearum*. From an earlier study (7), it seemed likely that a lectin might be involved in immobilizing of *E<sub>8</sub>* cells. Because lectins are commonly present at higher levels in plant seeds, we used apple seeds as a source of the presumed agglutinin.

A factor (AF) was indeed found that agglutinated *E<sub>8</sub>* much more effectively than *E<sub>9</sub>*, was heat-stable, and had a pH optimum for the agglutination reaction of 3.6 pH. The weaker agglutination of *E<sub>9</sub>* cells by AF requires an explanation. First, it is apparent that the AF binds with and precipitates the EPS present on the surface of the *E<sub>9</sub>* cells. This means that the EPS has receptor sites for AF and decreases the likelihood that the *E<sub>9</sub>* capsular polysaccharide protects the cells from agglutination by merely covering receptor sites on the surface of the cell. A more likely explanation is based on the observation that EPS on *E<sub>9</sub>* cells is present in two forms (15). One is an adherent, or tightly bound form, and the other is nonadherent, or loosely bound. At high concentrations AF would be removed from solution after interaction with nonadherent EPS. The residual AF could, in fact, bind to adherent EPS causing agglutination of the *E<sub>9</sub>* cells. However, if all of the available AF is precipitated by nonadherent EPS, the bacteria would no longer be agglutinated. This is analogous to the experiment in Figure 4 where EPS from *E<sub>9</sub>* cells is mixed with *E<sub>8</sub>* cells, and agglutination was inhibited.

AF was separated into higher (AF I) and lower (AF II) mol wt components by elution from a Bio-Gel P-100 column. Both components were capable of causing agglutination of *E<sub>8</sub>* cells. However, only AF II gave a precipitin reaction when subjected to electrophoresis in AFR-containing agar. Under the same conditions, AF I formed small precipitin arcs at the edge of the sample well. While the mol wt of AF I is not known, it is larger than AF II. Hence, it is possible that the active components in AF I were too large to enter the agar and interacted with AFR only at the edge of the sample well.

The relationship of AF I to AF II is not clear but some possibilities are suggested. First, it is possible that AF I and AF II are not related and represent 2 different molecules with different molecular weights. Second, AF I may be an aggregate of AF II. While aggregation of AF II could not be demonstrated in our experiments, its occurrence in seeds or during the extraction process cannot be excluded. Finally, since AF II binds to a number of negatively charged polymers, AF I may represent a series of complexes derived from AF II and negatively charged polymers released from the plant tissue during extraction. It is possible that complexes as large or larger than AF I (and, hence, lost during centrifugation) are the electron-dense granules observed in electron micrographs of *E<sub>8</sub>* cells agglutinated *in vivo* (10).

AF II elutes from the Bio-Gel P-100 column in the same fractions as ribonuclease indicating that it has an approximate mol wt of 12,600 daltons. It seems likely that the agglutination and precipitin activity in AF II resides in a protein for the following reasons. First, the molecule(s) with this activity possess a net positive charge at pH 3.6 and migrates rapidly (0.5 mm/min) in an electrical field. Second, the fractions from the P-100 column which

contain AF II contain neither carbohydrate nor uronic acid components which can be detected using colorimetric reagents. Third, the activities in AF II are destroyed by protease and trypsin. While it is impossible to exclude totally the presence of some carbohydrates in AF II, they were not detected by us.

Several observations suggest that both the precipitation and agglutination activities associated with AF II reside in the same molecule. First, both the precipitin and agglutination activity elute from Bio-Gel P-100 column in the same fraction. Second, both the agglutination and precipitin activities are unusually heat-stable. No detectable loss of activity occurs after heating at 121 C for 5 h. Third, treatment of AF II preparations with proteolytic enzymes results in a decrease of both precipitin and agglutination activities. Finally, when AF II is treated with EPS, both precipitin and agglutination activities are inhibited. Inasmuch as this inhibition results from the removal of AF II from solution, it is significant that both agglutination and precipitin reactions are completely inhibited at the same concentration of EPS.

It seems unlikely that the agglutination and precipitin activities in AF II reside in a lectin. AF II appears to lack the sugar-receptor specificity normally associated with lectins. While AF II interacts with a number of complex carbohydrates, it shows no specificity for the monosaccharide residues present in these polymers. It is capable of forming precipitin bands in agar with EPS from *E<sub>a</sub>*, carboxymethylcellulose, and sodium polygalacturonate. The common characteristic of these compounds is that they are polymers with a net negative charge.

We contend that the positively charged molecules of AF II interact with negatively charged polymers such as lipopolysaccharide and EPS on the surfaces of bacteria by charge-charge interaction. This interpretation is supported by the observation that poly-L-lysines can be used to replace AF II in both agglutination and precipitin assays and that poly-L-lysines are capable of removing the AF II receptor(s) from AFR preparations.

The pH optimum for the agglutination phenomena is considerably lower than would be expected to exist in normal healthy plant tissue. This study attempts to explain agglutination which occurs when bacterial cells are introduced into severely wounded plant tissue (6). It is not unlikely that the hydrogen-ion concentration would be sufficiently high in and near the wound area to permit the agglutination reaction to occur.

The fact that the activities associated with AF II might not reside in a lectin does not logically exclude AF II from playing a role in the *in situ* agglutination of *E. amylovora*. If the AF identified in this study is responsible for the *in situ* agglutination of *E. amylovora* cells, the following sequence of reactions might be proposed: When *E<sub>a</sub>* is introduced into cut apple tissue, it is agglutinated. The agglutinated cells are localized in the area of plant tissue where the pH is too low to permit bacterial growth (6) and they eventually die. This is in agreement with the observation that *E<sub>a</sub>* cells fail to multiply in apple tissue (10). When *E<sub>a</sub>* is introduced, AF reacts with and precipitates the nonadherent EPS associated with the *E<sub>a</sub>* cells. Since the *E<sub>a</sub>* cells are not agglutinated, they can move from the wounded tissue to areas of the plant where conditions are favorable for growth of the bacteria. Finally, if the proposed scheme of reactions is correct, AF might also play a role in the formation of the wilt symptoms caused by the virulent strain *E<sub>a</sub>*. In this case, AF would cause precipitation of nonadherent EPS in the vascular system and subsequently block water movement in that system. If AF plays a role in the precipitation of EPS, then we would expect the blockage to be limited to an area near the point of EPS introduction. That is, in the area where introduced *E<sub>a</sub>* cells are localized and conditions are presumably proper for AF to be active. This was demonstrated to be the case by Stoffl (20) and Suhayda and Goodman (22). They found that

if cut apple shoots were placed in a solution of EPS from *E<sub>a</sub>*, the shoots would form wilt symptoms. They also found that if wilted shoots were removed from the EPS solution and the first few millimeters of the cut end was removed, the shoots would regain their turgor. Further, Suhayda and Goodman (22) have presented ultrastructural evidence that the vascular elements in the first few millimeters near the cut end of the EPS-wilted shoot were uniformly occluded. Further, the occlusions induced by EPS and by the virulent *E<sub>a</sub>* bacteria were virtually indistinguishable. These results suggest that blockage of the vascular system was restricted to the site where the EPS was introduced and reflects the influence of an EPS-AF agglomerate. They also suggest that wilt symptom caused by *E. amylovora* is a reflection of the interaction between EPS of the pathogen and AF of the host.

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